DOI:
10.1016/j.bmcl.2016.05.006

Document Version
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PII: S0960-894X(16)30486-3
DOI: http://dx.doi.org/10.1016/j.bmcl.2016.05.006
Reference: BMCL 23860

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 10 December 2015
Revised Date: 20 April 2016
Accepted Date: 3 May 2016

Please cite this article as: Zhao, D-Y., Zhang, M-X., Dong, X., Hu, Y-Z., Dai, X-Y., Wei, X., Hider, R.C., Zhang, J-C., Zhou, T., Design and synthesis of novel hydroxypyridinone derivatives as potential tyrosinase inhibitors, *Bioorganic & Medicinal Chemistry Letters* (2016), doi: http://dx.doi.org/10.1016/j.bmcl.2016.05.006

Design and synthesis of novel hydroxypyridinone derivatives as potential tyrosinase inhibitors

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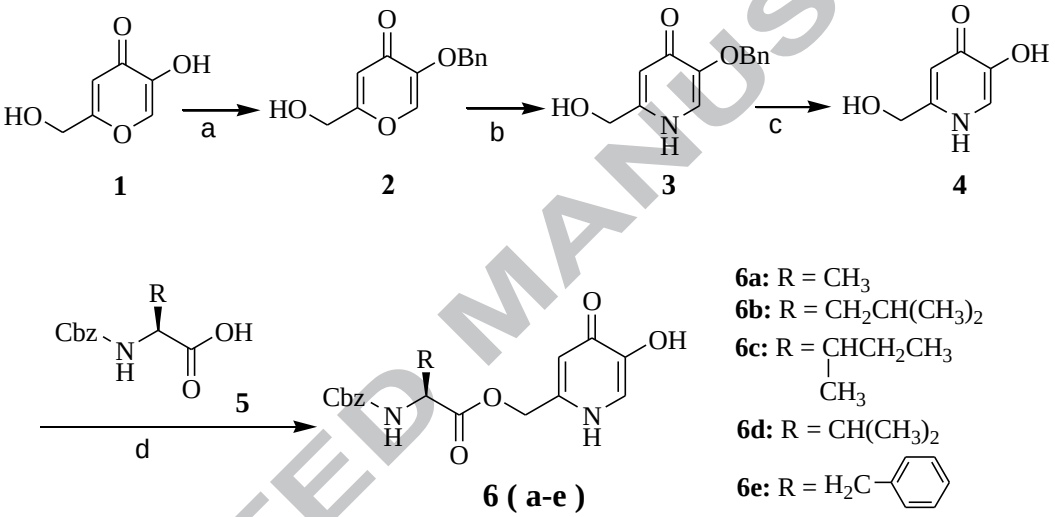
ABSTRACT: Two groups of novel hydroxypyridinone derivatives **6(a-e)** and **12(a-c)**, were designed as potential tyrosinase inhibitors, and synthesized using kojic acid as a starting material. The tyrosinase inhibitory activity of these two groups was demonstrated to be potent, especially compounds **6e** and **12a**, whose IC_{50} values for monophenolase activity were 1.95 μM and 2.79 μM, respectively. Both of these values are lower than that of kojic acid (IC_{50} = 12.50 μM). Compounds **6e** and **12a** were investigated for the inhibitory effect on diphenolase activity. The results showed that the inhibitory mechanism of these two compounds was reversible and that the inhibitory type was a competitive-uncompetitive mixed-type. The values of IC_{50} of **6e** and **12a** on the diphenolase activity of tyrosinase were determined to be 8.97 μM and 26.20 μM, respectively. The inhibitory constants (K_I and K_{IS}) of **6e** were determined as 17.17 μM and 22.09 μM, respectively; and the K_I and K_{IS} values of **12a** were 34.41 μM and 79.02 μM, respectively. Compound **6e** showed a greater ability to reduce copper and a stronger copper chelating ability than kojic acid.

Key words: 3-hydroxypyridinone; tyrosinase inhibitor; inhibitory mechanism

Tyrosinase (EC 1.14.18.1), a multifunctional copper-containing enzyme from the oxidase superfamily, is widely distributed in mammals, plants, microorganisms, and insects.[1] The active site of tyrosinase is well conserved among the different species. Six histidine residues, which are provided by a fourhelical bundle, coordinate the two copper ions, which serve as the major cofactors in the active site.[2,3] Tyrosinase can catalyze the first and rate-limiting step of melanin formation, namely the hydroxylation of *L*-tyrosine to *L*-3-(3,4-dihydroxyphenyl)-alanine (*L*-DOPA) (monophenolase activity) and also the subsequent oxidation of DOPA to dopaquinone (diphenolase activity).[4] Dopaquinone is highly reactive and can polymerize spontaneously to form melanin in a series of reaction pathways.[5] Therefore, tyrosinase plays an important role in the pigmentation of skin,[6,7] the browning of fruits and vegetables,[8,9] wound healing,[10] and cuticle formation in insects.[11,12] Tyrosinase is also involved in neuromelanin formation in the brain and neurodegeneration associated with Parkinson's disease.[13,14] Thus, tyrosinase inhibitors have attracted increasing attention. In principle, tyrosinase inhibitors have potential applications in the agricultural, cosmetic and pharmaceutical industry.

Numerous effects have been made to develop potent and safe tyrosinase inhibitors from natural materials and synthetic methods.[15-19] However, only a few are sufficiently potent for practical use and comply with the general safety regulations. Thus, there is a demand for novel tyrosinase inhibitors with superior activity together with reduced side effects. Kojic acid (**1**), a metabolic product of many species of *Aspergillus* and *Penicillium* moulds, has been used as an ingredient in cosmetics and as an anti-browning agent in foods that rapidly change colour by virtue of its appreciable anti-tyrosinase and antioxidant activities.[20-23] Thus, modification of kojic acid provides a potential route for superior tyrosinase inhibitors.[24-27] Kojic acid has been demonstrated to inhibit tyrosinase by chelating the copper ion normally present in the active site of tyrosinase. Using the principle of bioisosterism, we have changed the "O" at position-1 in pyranone ring to "NH", synthesizing a range of hydroxypyridinone-amino acid and hydroxypyridinone-dipeptide conjugates. Their tyrosinase inhibitory activity and mechanism of inhibition have been investigated.

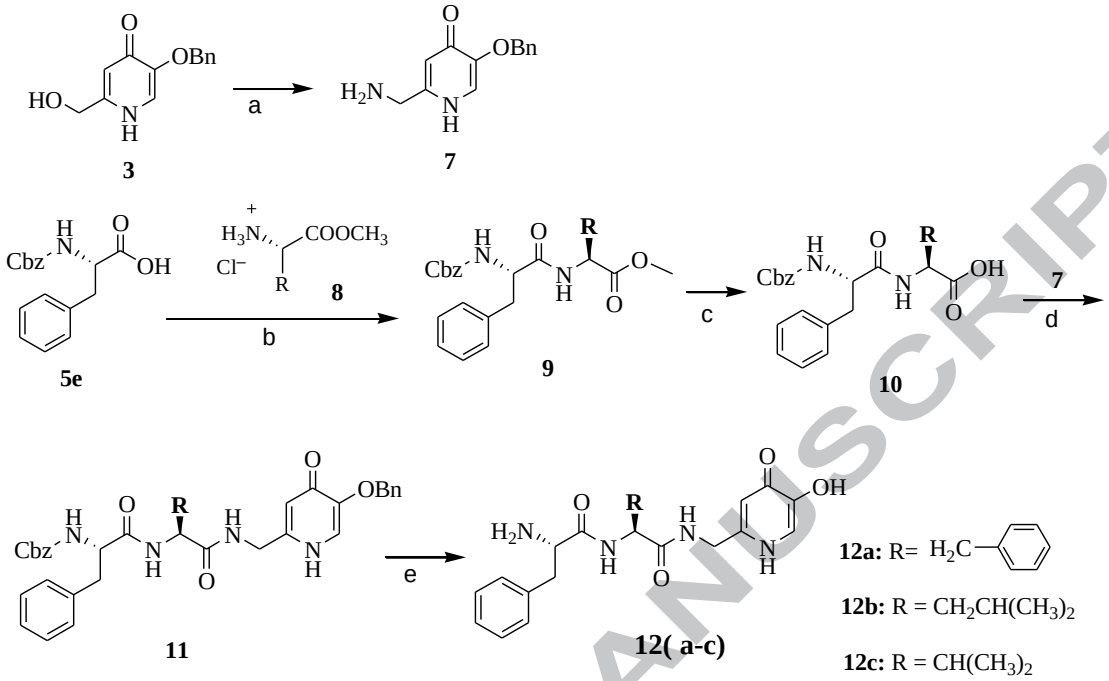
Hydroxypyridinone-*L*-amino acid conjugates **(6)** were synthesized starting from kojic acid (**1**) (Scheme 1). Benzylation of 5-hydroxy group in kojic acid was achieved by the reaction of kojic acid and benzyl chloride under basic condition to provide **2** in good yield (80%). Condensation of **2** with ammonia generated **3** (74%), which was then subjected to hydrogenation to remove benzyl group, providing compound **4** (82%). Coupling of **4** with Cbz-*L*-amino acid (**5**) via ester bond was carried out in the presence of EDC and DMAP in DMF at room temperature, yielding product **6**.[28]



Scheme 1. Reagents and conditions: (a) BnCl, MeOH/H_2O, 70^{o}C, 6h, 80 % yield; (b) aqueous NH_3 (37%), EtOH, reflux, overnight, 74 % yield; (c) MeOH/H_2O, H_2(30psi), 5% Pd/C, rt, 8h, 82 % yield; (d) Cbz-*L*-amino acid (**5**), EDC, DMAP, DMF, ice-bath, 2h; then rt overnight.

The synthetic route of hydroxypyridinone-dipeptide conjugates **(12)** is presented in Scheme 2. Compound **3** was treated with thionyl chloride, followed by the reaction with aqueous ammonia, providing amino group-containing compound **7**. N-benzyl oxygen carbonyl-*L*-phenylalanine (**5a**) reacted with methyl ester of *L*-amino acid (**8**) in the presence of HCTU, generating compounds **9**, which were then hydrolyzed to produce compounds **10**. The coupling of **10** with **7** was achieved in the presence of HCTU, yielding compounds **11**, which were subjected to hydrogenation in the presence of Pd/C catalyst, providing hydroxypyridinone-dipeptide conjugates **12**.[29]

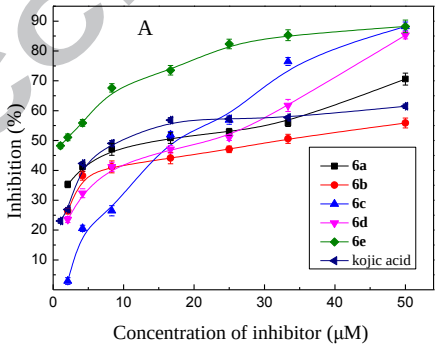
All the compounds have been fully characterized by ^{1}H NMR, ^{13}C NMR and HRMS.



Scheme 2. Reagents and conditions: (a) i) $SOCl_2$, rt, 18h; ii) methanol, aqueous ammonia (37%), rt 2h, then 40°C 8h, 69.5% yield; (b) DMF, HCTU, DIPEA, ice-bath 4-6h, over 93% yield; (c) MeOH/H_2O, LiOH, ice-bath 6-8h, hydrochloric acid; (d) DMF, HCTU, DIPEA, ice-bath 2h, then rt overnight; (e) H_2 (30psi), Pd/C (5%), BnCl, EtOAc/MeOH (1:1), rt 8-10h.

Inhibitory activity of hydroxypyridinone derivatives **6** and **12** on monophenolase activity of mushroom tyrosinase was investigated using *L*-tyrosine as a substrate.[30] The inhibitory effects on mushroom tyrosinase increased with the increase of concentrations of hydroxypyridinone derivatives (Figure 1). As shown in Figure 1A, the inhibitory activity of compounds **6** follows the order: **6e** > **6a** > **6d** > **6c** > **6b**. The IC_{50} values of compounds **6a-6e** were calculated to be 11.76, 28.71, 15.62, 12.48 and 1.95 μM respectively. Among these five hydroxypyridinone-amino acid derivatives, compound **6e** was found to exhibit the strongest inhibition against monophenolase activity of tyrosinase, being 6.4-fold more active than kojic acid (IC_{50} = 12.50 μM). Based on the data presented in Figure 1B, the IC_{50} values of compounds **12a-12c** were calculated to be 2.79, 6.93 and 14.26 μM, respectively. Compounds **12a** and **12b** are

4.5 and 1.8-fold more active than kojic acid against monophenolase activity of tyrosinase. Compound **12c** was found to have a lower inhibitory activity than kojic acid. Both copper-binding affinity and lipophilicity are important factors affecting the anti-tyrosinase activity. The copper-binding affinities of the hydroxypyridinone derivative prepared in the present study are anticipated to be similar due to the same chelating moieties and similar electronic effect of substituted group at position-6 in pyridine ring. For compounds **12,** the inhibitory effect decreased with the decrease of hydrophobicity (Clog P for **12a**, **12b** and **12c** are calculated to be 0.12, -0.04 and -0.57),[31] which is in good agreement with previously published results.[4,14] However, for compounds **6**, the inhibitory effects do not follow the order of hydrophobicity probably due to their high hydrophobicity (ClogP: **6a** 2.86; **6b** 4.17; **6c** 4.14; **6d** 3.64; **6e** 4.32). With such a high lipophilicity, these compounds are anticipated to readily enter the hydrophobic pocket of tyrosinase. In such cases, it is reasonably reckoned that the inhibitory activity mainly depends on the complimentary fit between the compound and active site of enzyme, which is affected by the substituting group and position on the pyridinone ring. In our previous study, it was found that hydroxypyridinone derivatives with a substitute at position-2 in pyridine ring had hardly anti-tyrosinase activity, although they have superior copper-binding affinity and higher lipophilicity than kojic acid.



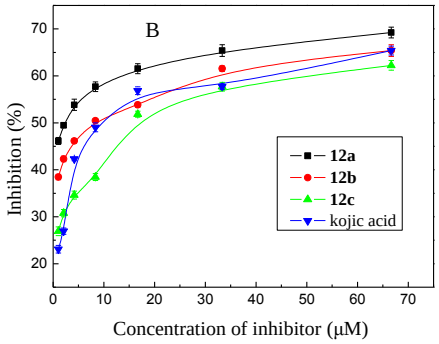
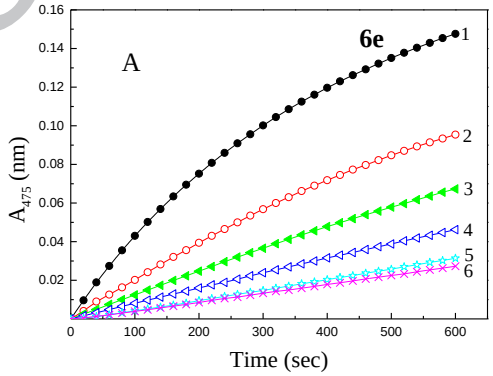


Figure 1. Inhibition on monophenolase activity of tyrosinase. (A) compounds **6**; (B) compounds **12.**

Inhibitory effect of **6e** and **12a** on diphenolase activity of mushroom tyrosinase was investigated using *L*-Dopa as a substrate. The kinetic courses of the oxidation of *L*-Dopa by mushroom tyrosinase in the presence of different concentrations of compound **6e** or **12a** were investigated. As shown in Figure 2, the formation of o-quinone increased with time. The absorbance values reduced with increasing concentration of compound **6e** and **12a**, indicating an increase of inhibitory effect. Under the same conditions, the inhibitory effect of compound **6e** was better than that of compound **12a**. In the cases of both **6e** and **12a**, the increasing rate of o-quinone formation became slower with increasing reaction time, indicating that the inhibitory effect decreased. In addition, the reaction process catalyzed by the diphenolase activity of tyrosinase had no lag time.



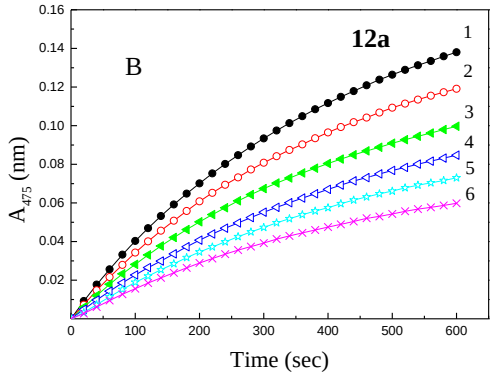


Figure 2. Inhibition kinetics on diphenolase activity of tyrosinase. (A) **6e**; (B) **12a**. The concentrations of inhibitors (**6e** and **12a**) for curves 1-6 were 0.00, 4.17, 8.33, 16.67, 25.00 and 33.33μM, respectively.

As indicated in Figure 3, the relative activity of enzyme decreased with increasing concentration of inhibitor, indicating that the activity of enzyme was inhibited in a dose dependent manner. The IC_{50} values of compounds **6e** and **12a** were calculated to be 8.97 and 26.20 μM, respectively. Thus, **6e** is more effective than **12a** in the inhibition on diphenolase activity of tyrosinase.

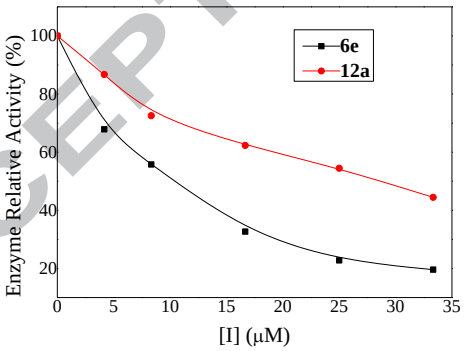


Figure 3. Inhibitory effect of **6e** and **12a** on the diphenolase activity of tyrosinase.

The inhibitory mechanism of **6e** and **12a** on mushroom tyrosinase was investigated using *L*-DOPA as a substrate. For both compounds, investigation on the relationship between enzyme activity and its concentration in the presence of compound **6e** and **12a** indicated that the plots of the remaining enzyme activity versus

the concentration of enzyme at different inhibitor concentrations gave a family of straight lines, which all passed through the origin (Figure 4). This result is similar to that reported by Chen *et al.*[32] Increase of inhibitor concentration resulted in descent of the slope of the line, indicating that the presence of inhibitor resulted in the inhibition of enzyme activity.[33] Thus, the inhibition of both compounds **6e** and **12a** on diphenolase activity of tyrosinase is reversible.

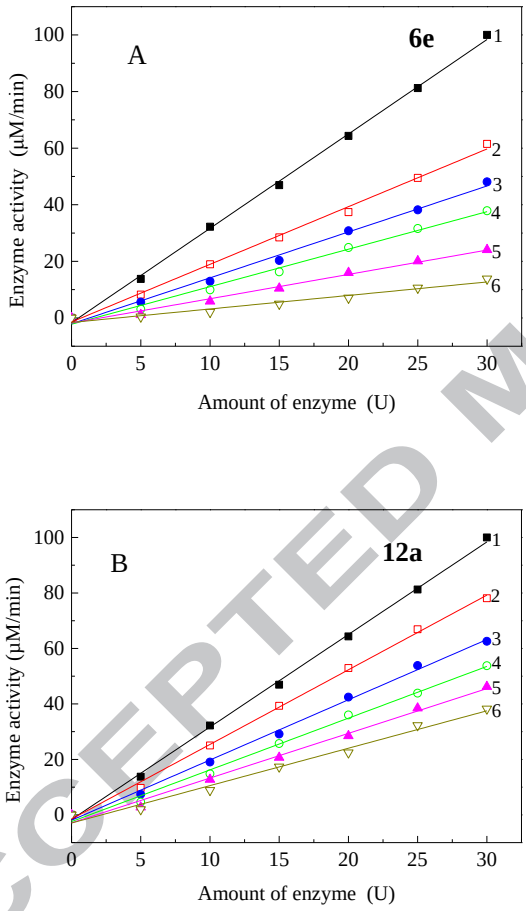
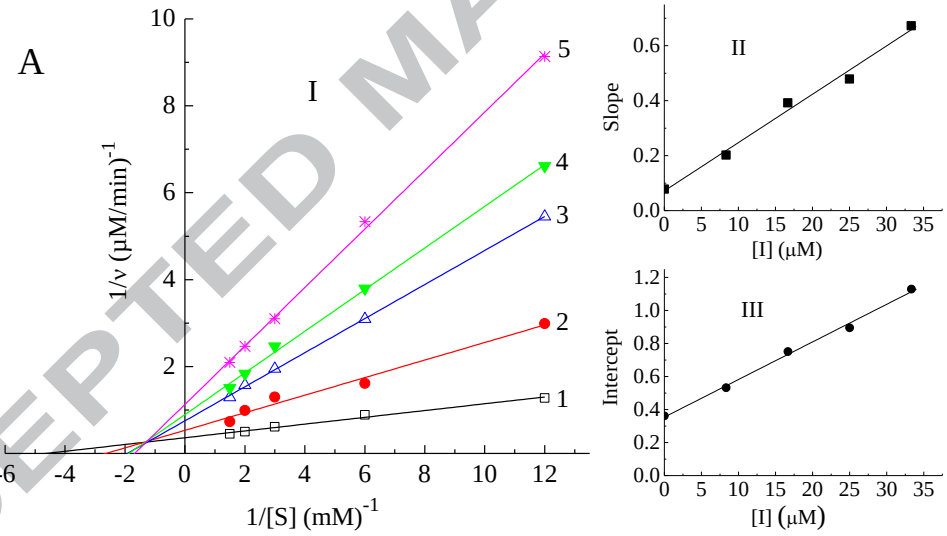


Figure 4. Determination of the inhibitory mechanism of **6e** (A) and **12a** (B) on mushroom tyrosinase. The concentrations of inhibitors for curves 1-6 were 0.00, 4.17, 8.33, 16.67, 25.00 and 33.33 μM, respectively.

The kinetic data of the inhibition of *L*-DOPA oxidation by **6e** and **12a** were expressed in Lineweaver-Burk double-reciprocal plots (Figure 5A-I, B-I).[33] The plots of 1/v versus 1/[S] gave a group of straight lines with different slopes that intercept in the second quadrant, indicating that both compounds **6e** and **12a** can bind not only

with free enzyme but also with the enzyme−substrate complex, namely, both **6e** and **12a** were competitive−uncompetitive mixed type inhibitors. The equilibrium constant of inhibitor for binding with free enzyme (K_I) was obtained from a plot of slope (K_m/V_m) versus the concentration of the inhibitor (Figure 5A-II and B-II), and with enzyme−substrate complex (K_{IS}) was obtained from a plot of the vertical intercept ($1/V_m$) versus the concentration of the inhibitor (Figure 5A-III and B-III). The K_I and K_{IS} values of **6e** were determined to be 17.17 μM and 22.09 μM, respectively. The inhibitor constants (K_I and K_{IS}) of **12a** were determined as 34.41 μM and 79.02 μM, respectively. In both cases, the K_{IS} value is larger than the K_I value, indicating that the affinity of the inhibitors for free enzyme is greater than that for the enzyme-substrate complex.



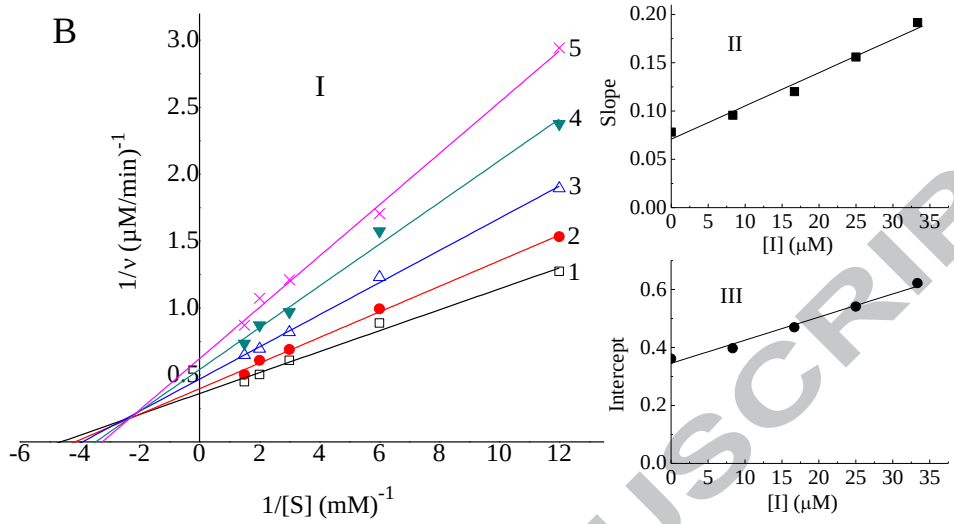


Figure 5. Lineweaver–Burk plots (A-I and B-I) of mushroom tyrosinase with *L*-DOPA as a substrate in the presence of **6e** (A) and **12a** (B). **A**-II and B-II represent the plot of slope versus the concentration of **6e** and **12a** for determining the inhibition constants K_I. A-III and B-III represent the plot of intercept versus the concentration of **6e** and **12a** for determining the inhibition constants K_{IS}.

In order to further investigate the inhibitory mechanism of hydroxypyridinone derivatives, copper reduction capacity[34] and copper chelating ability[35] of compound **6e** were determined. The capacity of the reducing cupric ion to cuprous ion by **6e** at different concentrations is shown in Figure 6A. The increases in absorbance value at 483nm indicate the formation of cuprous ion. The absorbance increased with increasing concentration of compound **6e** up to 0.15 mM, thereafter the absorbance remained unchanged, indicating that all cupric ions were reduced to cuprous ion. In the case of kojic acid, the absorbance at 483nm increased with the increase of its concentration until 0.3 mM, indicating that compound **6e** possesses a stronger copper reducing capability than kojic acid. It is well known that tyrosinase exists in three isoforms, namely, oxy-tyrosinase [Cu(II)Cu(II)O_2], met-tyrosinase [Cu(II)Cu(II)], and deoxy-tyrosinase [Cu(I)Cu(I)].[36] Met-tyrosinase is reduced by reductant to deoxy-tyrosinase, which is then oxidized by oxygen, forming oxy-tyrosinase capable of catalyzing mono- or diphenol oxidation.[37] Copper in the active site of tyrosinase

plays a key role in browning reaction. Reduction of cupric ion to cuprous ion at the active site of tyrosinase by compound **6e** could convert tyrosinase into the deoxy form. Thus, it is suggested that compound **6e** could inhibit the dopachrome formation by reducing met-tyrosinase to deoxy-tyrosinase.

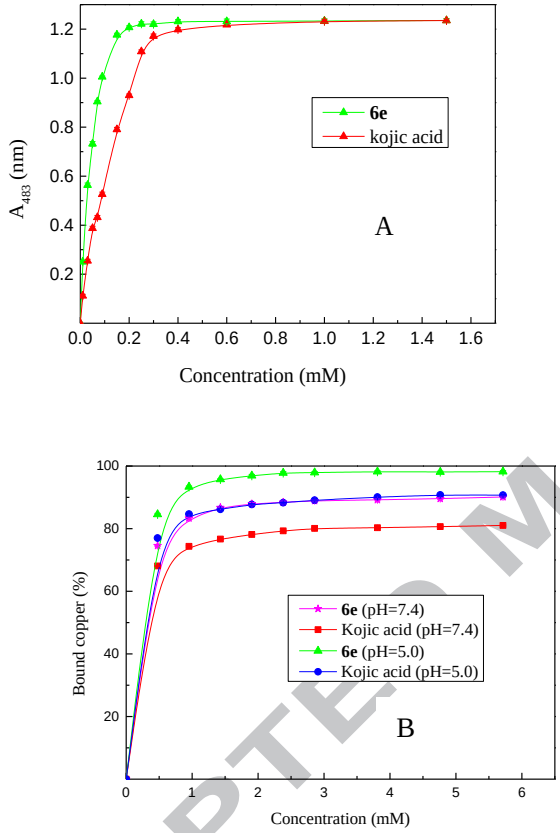


Figure 6. Copper reducing capacity (A) and copper chelating ability (B) of compound **6e** and kojic acid.

As shown in Figure 6B, at low concentrations (<1mM), the copper chelating ability of compound **6e** and kojic acid both increased with increasing concentration ($P < 0.05$), after which the increasing rate rapidly slowed down and then reached a maximum. For compound **6e**, at pH 5.0 and 7.4, copper chelating abilities were determined to be 91.34±0.28% and 83.34±0.29% at 0.952 mM, and 97.52±0.31% and 88.92±0.33% at 2.857mM, respectively; while for kojic acid, at pH 5.0 and 7.4, they were 82.64±0.25% and 74.35±0.27% at 0.952 mM, reaching a maximum of 90.10±0.25% and 80.08±0.22% at 3.81mM, indicating superior copper chelating

ability of **6e** to that of kojic acid. Tyrosinase contains a coupled binuclear copper center in its active site. The two cupric ions, bound with three histidine residues, are directly involved in the different catalytic activities. When compound **6e** enters the active site of the tyrosinase, it could outcompete with the histidine residues for the binding of cupric ion, thereby resulting in a loss of activity. The copper chelating activity of **6e** was in accordance with its tyrosinase inhibitory activity (Figure 1). Therefore, copper chelation of **6e** is one of the important mechanisms for the inhibition of tyrosinase.

In order to understand the interaction mode of inhibitor binding to tyrosinase, the molecular docking of compound **6e** to *Agaricus bisporus* tyrosinase[38] was carried out using Accelrys Discovery Studio 2.5 software. As shown in Figure 7, the hydroxypyridinone ring of compound **6e** can insert into the bottom of active pocket in tyrosinase, the 3-hydroxy group and 4-carbonyl group coordinate with copper ion. Two benzyl groups stretch out with a T shape, forming a stable combined conformation via hydrophobic interaction with hydrophobic area in the active pocket of tyrosinase, indicating a good deal of complimentary fit between compound **6e** and active site of tyrosinase.

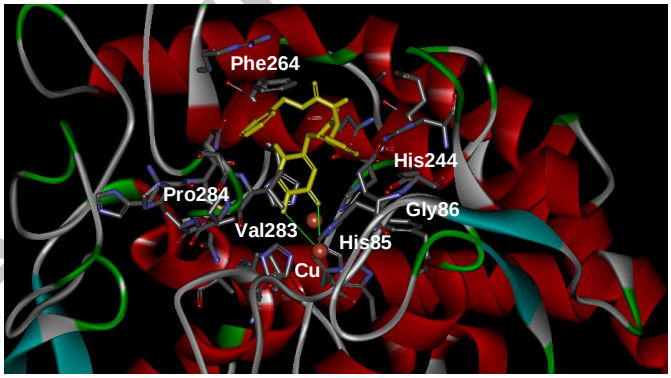


Figure 7. Structure for the interaction of compound **6e** with tyrosinase.

In summary, a range of hydroxypyridinone derivatives were synthesized in the present study. Among them, **6e** was found to exhibit the highest tyrosinase inhibitory activity. **6e** exhibited reversible and mixed type inhibition on mushroom tyrosinase.

Chelating copper at the active site of tyrosinase is an important mechanism for the inhibition of tyrosinase. Thus it could find application in medicine, cosmetic and agriculture areas.

Acknowledgement

The authors acknowledge the financial support for this work provided by Science Technology Department of Zhejiang Province of China (No. 2013C24006) and the National Natural Science Foundation of China (No. 20972138).

References and notes

1. Song, K. K.; Huang, H.; Han, P.; Zhang, C. L.; Shi, Y.; Chen, Q. X. *Biochem. Biophys. Res. Commun.* **2006**, *342*, 1147.
2. Siegbahn, Per E. M. *J. Biol. Inorg. Chem.* **2003**, *8*, 567.
3. Sendovski, M.; Kanteev, M.; Ben-Yosef, V. S.; Adir, N.; Fishman, A. *J. Mol. Biol.* **2011**, *405*, 227.
4. Li, D. F.; Hu, P. P.; Liu, M. S.; Kong, X. L.; Zhang, J. C.; Hider, R. C.; Zhou, T. *J. Agric. Food Chem.* **2013**, *61*, 6597.
5. Seo, S. Y.; Sharma, V. K.; Sharma, N. *J. Agric. Food Chem*. **2003**, *51*, 2837.
6. Olivares, C.; Solano, F. *Pigment Cell Melanoma Res.* **2009**, *22*, 750.
7. Jimbow, K.; Park, J. S.; Kato, F.; Hirosaki, K.; Toyofuku, K.; Hua, C.; Yamashita, T. *Pigment Cell Res.* **2000**, *13*, 222.
8. Kim, Y. J.; Uyama, H. *Cell Mol. Life Sci.* **2005**, *62*, 1707.
9. Rescigno, A.; Sollai, F.; Pisu, B.; Rinaldi, A.; Sanjust, E. *J. Enzyme Inhib. Med. Chem.* **2002**, *4*, 207.
10. Lai, S. C.; Chen, C. C.; Hou, R. F. *J. Med. Entomol.* **2001**, *38*, 836.
11. Guerrero, A.; Rosell, G. *Curr. Med. Chem.* **2005**, *4*, 461.
12. Gorman, M.J.; An, C.; Kanost, M. R. *Insect Biochem. Mol. Biol.* **2007**, *37*, 1327.
13. Xu, Y.; Stokes, A. H.; Freeman, W. M.; Kumer, S. C. *Mol. Brain Res.* **1997**, *45*, 159.

14. Gaeta, A.; Molina-Holgado, F.; Kong, X. L.; Salvage, S.; Fakih, S.; Francis, P. T.; Williams R. J.; Hider, R. C. *Bioorg. Med. Chem.*, **2011**, *19*, 1285.
15. Solano, F.; Briganti, S.; Picardo, M.; Ghanem, G. *Pigment Cell Res.* **2006**, *19*, 550.
16. Ansari, M.; Etelaei, F.; Sharififar, F. *Int. J. Green Pharm.* **2011**, *5*, 103.
17. Ding, H. Y.; Chou, T. H.; Liang, C. H. *Food Chem.* **2010**, *123*, 254.
18. Ha, Y. M.; Park, Y. J.; Kim, J. A.; Park, D.; Park, J. Y.; Lee, H. J.; Lee, J. Y.; Moon, H. R.; Chung, H. Y. *Eur. J Med. Chem.* **2012**, *49*, 245.
19. Cui, Y.; Liang, G.; Hu, Y. H.; Shi, Y.; Cai, Y. X.; Gao, H. J.; Chen, Q. X.; Wang Q. *J. Agric. Food Chem.* **2015**, *63*, 716.
20. Ahn, S. M.; Rho, H. S.; Baek, H. S.; Joo, Y. H.; Hong, Y. D.; Shin, S. S.; Park, Y.-H.; Park, S. N. *Med. Chem. Lett.* **2011**, *21*, 7466.
21. Mitani, H.; Koshiishi, I.; Sumita, T.; and Imanari, T. *Eur. J. Pharmacol.* **2001**, *411*, 169.
22. Kitao, S.; Shimaoka, Y. and Sekine, H. Japan Kokai Tokkyo Koho, 56872 (Mar. 1, 1994).
23. Higa, Y. and Nakajima, K. Japan Kokai Tokkyo Koho, 319473 (Dec. 25, 1989).
24. Choi, Y. J.; Rho, H. S.; Baek, H. S.; Hong, Y. D.; Joo, Y. H.; Shin, S. S.; Kim, J. M. *Bull. Korean Chem. Soc.* **2014**, *35*, 3647.
25. Kwak, S.Y.; Choi, H. R.; Park, K. C.; Lee, Y. S. *J. Pept. Sci.*, **2011**, *17*, 791.
26. Lima, C. R.; Silva, J. R. A.; Carvalho Cardoso, E. D. T.; Silva, E. O.; Lameira, J.; do Nascimento, J. L. M.; Brasil, D. D. B. ; Alves, C. N. *Molecules* **2014**, *19*, 9591.
27. Lachowicz, J. I.; Nurchi, V. M.; Crisponi, G.; Pelaez, M. D. J.; Rescigno, A.; Piotr Stefanowicz, P.; Cal, M.; Szewczuk, Z. *J. Inorg. Biochem.* **2015**, *151*, 36.
28. General procedure for preparation of **6**. To a solution of compound **4** (5 mmol) and Cbz-*L*-amino acid (**5)** (5.5 mmol) in DMF (20 mL) was added 1-ethyl-(3-dimethyllaminopropyl) carbodiimine hydrochloride (EDC, 5.5 mmol) and 4-dimethylaminopyridine (DMAP, 0.55 mmol). The resulting solution was stirred on an ice bath for 2h, then at room temperature overnight. After removal of the solvent, the residue was dissolved in CH_2Cl_2 (60mL), and washed with saturated $NaHCO_3$ solution twice followed by brine, and dried over anhydrous Na_2SO_4. After filtration, the filtrate was concentrated, then the residue was purified by silica gel column chromatography ($MeOH/CH_2Cl_2$ 1:10), providing compounds **6** as off-white solids. The silica gel column must be pretreated with

maltol to remove iron completely. Data for **6e**: Yield 71.3%. ^{1}H NMR (500MHz, DMSO-d_6) δ: 2.92-3.11 (m, 2H, CH_2), 4.39 (m, 1H, CH), 4.98 (s, 2H, CH_2), 5.21 (s, 2H, CH_2), 7.15 (s, 1H, C3-H in pyridinone), 7.20-7.35 (m, 10H, Ph), 7.89 (d, *J*=6.5Hz, 1H, NH), 8.08 (s, 1H, C6-H in pyridinone). ^{13}C NMR (125MHz, DMSO-d_6) δ: 36.80, 55.93, 62.00, 65.98, 112.39, 127.00, 127.30, 128.03, 128.26, 128.68, 128.77, 129.59, 137.30, 137.67, 142.81, 145.32, 156.46, 161.71, 171.73. ESI-HRMS: *m/z*, calcd for $C_{23}H_{23}N_2O_6$ ($[M+H]^+$) 423.1551, found 423.1542.

29. General procedure for preparation of **12**. To a suspension of compound **11** (2 mmol), BnCl (2.4 mmol) in methanol (20 mL)/ethyl acetate (20 mL) was added 5 % Pd/C (5% weight of compounds **11**). Hydrogenation was carried out under 30 psi H_2 for 8 h at room temperature. After filtration to remove the catalyst, the filtrate was concentrated, small amount of diethyl ether was added, allowed to stand in a fridge. Hydrochloride salts of compounds **12** were obtained as white powder by filtration. Data for **12a**: Yield 86.5%. ^{1}H NMR (500MHz, DMSO-d_6) δ: 2.95-3.13 (m, 4H, CH_2, CH_2), 4.04 (m, 1H, CH), 4.37-4.48 (m, 2H, CH_2), 4.54 (m, 1H, CH), 7.13 (s, 1H, C3-H in pyridinone), 7.19-7.31 (m, 10H, Ph), 8.10 (s, 1H, C6-H in pyridinone), 8.26 (br, 3H, NH_3^+), 9.01 (t, *J*=6.0Hz, 1H, NH), 9.05 (d, *J*=8.0Hz, 1H, NH). ^{13}C NMR (125MHz, DMSO-d_6) δ: 19.06, 37.23, 37.63, 53.87, 55.18, 56.53, 111.12, 126.32, 126.92, 127.52, 128.66, 128.90, 129.69, 130.08, 135.33, 137.92, 146.66, 146.84, 161.83, 168.50, 171.49. ESI-HRMS: *m/z*, calcd for $C_{24}H_{27}N_4O_4$ ($[M+H]^+$) 435.2027, found 435.2014.

30. Determination of inhibitory activity of hydroxypyridinone derivatives on mushroom tyrosinase: *L*-tyrosine (2 mM) was used as the substrate for the monophenolase activity assay and *L*-DOPA (0.5 mM) was used as the substrate for the diphenolase activity assay. *L*-tyrosine (1 mL) or L-DOPA (1 mL), phosphate buffer (1.8 mL, pH 6.8) and different concentrations of inhibitor (0.1 mL in DMSO) were mixed and incubated at 30 ^{o}C. Then, an aqueous solution of mushroom tyrosinase (0.1 mL, 200U/mL) was added to the above solution and mixed quickly. The reaction system (totally 3mL) was incubated at 30 ^{o}C for 10 min. Absorption and kinetic measurements were carried out on a Shimadzu UV3600 UV-Vis spectrophotometer at 475nm. Controls containing the same amount of DMSO without inhibitor, were routinely carried out. All the measurements were performed in triplicate. The inhibition rate of tyrosinase was calculated according to the following formula:

Inhibition rate (%) = [1-(OD_3-OD_4) / (OD_1-OD_2)] × 100

where OD_1 was the absorbance value without inhibitor, OD_2 was the absorbance value without substrate and inhibitor, OD_3 was the absorbance value of the experimental group, OD_4 was the absorbance value without substrate.

31. ClogP value is an important parameter to evaluate the lipophilicty of a non-charged compound. The ClogP values in this paper were calculated using the following web software: http://www.molinspiration.com/cgi-bin/properties.
32. Chen, L. H.; Hu, Y. H.; Song, W.; Song, K. K.; Liu, X.; Jia, Y. L.; Zhuang, J. X.; Chen, Q. X. *J. Agric. Food Chem.* **2012**, *6*, 1542-1547.
33. Li, Z. C.; Chen, L. H.; Yu, X. J.; Hu, Y. H.; Song, K. K.; Zhou, X. W.; Chen, Q. X. *J. Agric. Food Chem.* **2010**, *58*, 12537-12540.
34. Chen, J. S.; Wei, C. I.; Marshall, M. R. *J. Agric. Food Chem.* **1991**, *39*, 1897–1901.
35. The copper chelating capacity of compound **6e** was determined according to a reported method (Nirmal, N. P.; Benjakul, S. *LWT-Food Sci. Technol.* **2011**, *44*, 924–932.) with a slight modification. $CuSO_4$ (1 mM) and tetramethylmurexide (TMM) (5 mM) were prepared in hexamine buffer (10 mM, pH 5.0 and 7.4) containing 10 mM KCl. Chelator solutions (1-12mM) were prepared by diluting the stock solution (in DMF) with the same buffer. $CuSO_4$ (l.0 mL) was mixed with a chelator solution (1.0 mL), followed by the addition 0.1 mL of TMM. The absorbance of the resulting reaction mixture was recorded at 460 nm and 530 nm after incubation for 10 min at room temperature. According to the calculated absorbance ratios of A_{460}/A_{530}, the corresponding free cupric ion concentrations were obtained from a standard curve of free cupric ion concentration (0–0.1 mM) vs absorbance ratio. The difference between the total cupric ion and the free cupric ion concentrations indicated the concentration of chelated cupric ion. Assays were performed in triplicate.
36. Likhitwitayawuid, K. *Curr. Sci.* **2008**, *94*, 44.
37. Winkler, M. E.; Lerch, K.; Solomon, E. I. *J. Am. Chem. Soc.* **1981**, *103*, 7001.
38. The X-ray crystal structure of *Agaricus bisporus* tyrosinase (PDB ID: 2Y9X) was retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb).

Graphical Abstract

Design and Synthesis of Novel Hydroxypyridinone Derivatives as Potential Tyrosinase Inhibitors

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